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# NF-κB p105 is a target of IκB kinases and controls signal induction of Bcl-3–p50 complexes

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**The NF-κB precursor p105 has dual functions: cytoplasmic retention of attached NF-κB proteins and generation of p50 by processing. It is poorly understood whether these activities of p105 are responsive to signalling processes that are known to activate NF-κB p50–p65. We propose a model that p105 is inducibly degraded, and that its degradation liberates sequestered NF-κB subunits, including its processing product p50. p50 homodimers are specifically bound by the transcription activator Bcl-3. We show that TNFα, IL-1β or phorbolester (PMA) trigger rapid formation of Bcl-3–p50 complexes with the same kinetics as activation of p50–p65 complexes. TNF-α-induced Bcl-3–p50 formation requires proteasome activity, but is independent of p50–p65 released from IκBα, indicating a pathway that involves p105 proteolysis. The IκB kinases IKKα and IKKβ physically interact with p105 and inducibly phosphorylate three C-terminal serines. p105 is degraded upon TNF-α stimulation, but only when the IKK phospho-acceptor sites are intact. Furthermore, a p105 mutant, lacking the IKK phosphorylation sites, acts as a super-repressor of IKK-induced NF-κB transcriptional activity. Thus, the known NF-κB stimuli not only cause nuclear accumulation of p50–p65 heterodimers but also of Bcl-3–p50 and perhaps further transcription activator complexes which are formed upon IKK-mediated p105 degradation.**

*Keywords*: kinases/oncogene/proteasome/transcription activation

## Introduction

The members of the NF-κB family of transcription factors play an essential role in a number of physiological processes including inflammatory, stress and immune responses, apoptosis and cellular proliferation (Baeuerle and Henkel, 1994; Wulczyn *et al*., 1996; Barnes and Karin, 1997). In vertebrates, the family consists of the five members p50, p65(RelA), p52, c-Rel and RelB which share a conserved DNA-binding and dimerization domain and form various homo- and heterodimers. They are retained in the cytoplasm by tight association with members of a co-evolved IκB protein family. IκBs share a conserved domain of six or seven ankyrin repeats. Such a structure is also found in the C-terminal sequences of p105 and p100, which are the precursor molecules for p50 and p52, respectively (Baeuerle and Baltimore, 1996; Baldwin, 1996; May and Ghosh, 1997). *In vitro* studies demonstrated that the IκB family members have distinguishable specificities towards NF-κB/Rel proteins and can be divided into three groups: the precursor proteins p100 and p105 can bind efficiently to all mammalian NF-κB factors; IκBα, IκBβ and IκBε strongly prefer dimers containing p65 or c-Rel. In contrast, Bcl-3 has a strong preference towards p50 or p52 homodimers (Wulczyn *et al*., 1996; May and Ghosh, 1997).

In response to a variety of stimuli, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), bacterial lipopolysaccharides (LPS) or UV light, NF-κB p50–p65 is released from the small IκB proteins IκBα, β and ε, translocates to the nucleus and activates transcription. The release is caused by subsequent phosphorylation, ubiquitination and proteasomal degradation of the small IκBs (Israel, 1995). Signal-induced phosphorylation occurs at serine residues in a conserved DSGψXS motif in their N-terminal sequences by a 700 kDa IκB kinase complex (Maniatis, 1997). This complex contains two IκB kinases, IKKα and IKKβ, as well as structural components, IKKγ/NEMO and IKAP (DiDonato *et al*., 1997; Mercurio *et al*., 1997, 1999; Regnier *et al*., 1997; Woronicz *et al*., 1997; Zandi *et al*., 1997; Cohen *et al*., 1998; Rothwarf *et al*., 1998; Yamaoka *et al*., 1998). The IκB degradation mechanism has been studied most intensively for IκBα. Phosphorylation of IκBα at serines 32 and 36 in the DSGψXS motif marks the inhibitor for ubiquitination at lysines 21 and 22; ubiquitination leads to subsequent degradation by the proteasome (Alkalay *et al*., 1995; Brockman *et al*., 1995; Brown *et al*., 1995; Chen *et al*., 1995; Scherer *et al*., 1995; Traenckner *et al*., 1995; Whiteside *et al*., 1995; Baldi *et al*., 1996; DiDonato *et al*., 1996; Rodriguez *et al*., 1996; Roff *et al*., 1996). N-terminal IκBα sequences containing these serine and lysine residues are sufficient to act as an inducible destruction box when fused to heterologous proteins (Wulczyn *et al*., 1998). Even in the absence of stimuli, the IκBα protein is unstable and is turned over rapidly. This continuous basal degradation is controlled by a different mechanism, as it requires neither the IKK phosphorylation sites nor ubiquitin attachment, but it is also carried out by the proteasome (Krappmann *et al*., 1996).

Proteolytic processing of p105 to p50, resulting in the selective degradation of its C-terminal sequences, has been analysed in mammalian and yeast cells and appears to be a largely constitutive process; in most cells, p105 and p50 are produced in nearly stoichiometric amounts (Naumann *et al*., 1993b; Lin and Ghosh, 1996; Lin

*et al*., 1998). p105 processing involves ubiquitination and proteasomal destruction (Palombella *et al*., 1994). C-terminal to the p50 moiety, p105 contains a glycine-rich region which is required for processing in mammalian cells and may be the target of an endoprotease (Lin and Ghosh, 1996). However, this sequence is apparently not required for p105 processing in yeast cells (Sears *et al*., 1998). Recently, Lin *et al*. (1998) reported that p105 processing by the proteasome occurs co-translationally and that p50 and p105 do not exhibit a classical product–precursor relationship. The mechanism that determines whether processing occurs co-translationally or also post-translationally (Belich *et al*., 1999) will have to be analysed in future studies.

It is currently not understood under which conditions processing can be induced by NF-κB-activating signals. However, treatment of cells with various NF-κB-inducing agents results in phosphorylation of endogenous p105 (Neumann *et al*., 1992; Mellits *et al*., 1993; Naumann and Scheidereit, 1994). Signal-induced enhanced processing to p50 has been proposed (Mellits *et al*., 1993; Mercurio *et al*., 1993; Naumann and Scheidereit, 1994; MacKichan *et al*., 1996), but the observed changes in the p50/p105 ratios upon stimulation were rather modest. Very recently, Belich *et al*. (1999) reported that p105 is completely degraded without giving rise to the processing product p50, when the MAP3K Tpl-2 is overexpressed. Although overexpressed Tpl-2 interacts with p105, it does not phosphorylate p105 directly (Belich *et al*., 1999) and rather acts upstream of NIK (Lin *et al*., 1999).

p50 or p52 homodimers generated by processing of p105 or p100 are the known targets for the IκB homologue Bcl-3. Bcl-3 is a unique IκB member, since it is most abundant in the nucleus and is not degraded upon activation of NF-κB-stimulating pathways. Bcl-3 can have different effects on p52 or p50 binding to DNA, depending on its phosphorylation status, concentration or interaction with nuclear co-factors (Wulczyn *et al*., 1992; Bours *et al*., 1993; Nolan *et al*., 1993; Bundy and McKeithan, 1997; Dechend *et al*., 1999). The interaction of Bcl-3 with p50 or p52 homodimers can result in their dissociation from DNA. Since neither p50 nor p52 contain transactivation domains, it has been proposed that Bcl-3 thus may antagonize p50-mediated inhibition (Franzoso *et al*., 1992). Alternatively, Bcl-3 can form ternary complexes with p50 or p52 homodimers bound to DNA and act as a transcription activator (Bours *et al*., 1993; Fujita *et al*., 1993; Pan and McEver, 1995; Hirano *et al*., 1998). Transcription activation requires the presence of N- and C-terminal proline- and serine-rich domains (Bours *et al*., 1993). The activation potential of Bcl-3–p50 complexes can be stimulated further by interaction of Bcl-3 with the histone acetylase Tip60 (Dechend *et al*., 1999).

Ectopic overexpression of Bcl-3 in the murine thymus caused an enhancement of the DNA-binding activity of p50 homodimers (Caamano *et al*., 1996). Similarly, overexpression of Bcl-3 in pro-B cell lines resulted in augmented amounts of p50 homodimers in the nucleus. This involves the liberation by Bcl-3 of p50 homodimers from cytosolic p105–p50 complexes, apparently without causing increased processing of p105 (Watanabe *et al*., 1997).

We now provide evidence that Bcl-3–p50 complex formation is induced by a variety of NF-κB-activating agents. The generation of Bcl-3–p50 complexes was dependent on the enzymatic activity of the proteasome but independent of p50–p65 release, suggesting that p50 homodimers are liberated through enhanced processing or degradation of p105. We demonstrate that activation of the IκB kinase complex results in phosphorylation of p105 at C-terminal residues. Phosphorylation was abolished when serines 921, 923 and 932 were mutated. These residues were phosphorylated with similar efficiency as serines 32 and 36 of IκBα. Both IKKs associated with p105; this interaction was conferred by the C-terminal sequence containing the phosphorylation sites. The same region was required for complete degradation rather than processing of p105 after TNF-α stimulation and induced degradation could be blocked when the three serines were mutated. A p105 mutant devoid of its C-terminal destruction box, just like N-terminally truncated IκBα, acts as a super-repressor molecule, by inhibiting IKK-induced NF-κB. Thus, IKK-induced degradation of p105 provides a means to activate Bcl-3–p50 complexes rapidly in parallel to p50–p65 heterodimers released by degradation of the small IκBs.

## Results

### *Activation of p50 homodimers bound to Bcl-3 in response to NF-κB-activating agents*

To analyse cellular Bcl-3–p50, we established a method which allows detection of these complexes in the background of other NF-κB/Rel activities. Bcl-3-bound NF-κB/Rel proteins were revealed by electrophoretic mobility shift assay (EMSA) with detergent eluates of complexes precipitated with a Bcl-3-specific antibody (IP-shift assay). As a control, whole-cell extracts were immunoprecipitated with an IκBα-specific antibody. The proteins eluted from the IκBα antibody pellet contained a DNA-binding complex consisting of p50–p65, as expected (Figure 1A). This complex (lane 2) was not observed when the peptide antigen was added in the immunoprecipitation step (lane 1) and could be supershifted or inhibited, respectively, by anti-p50 or anti-p65 antibodies added to the DNA-binding reaction (lanes 4 and 5). The supershift was abolished when the antibody was blocked with its specific peptide (lane 3). In a similar fashion, a Bcl-3 antibody specifically precipitated a DNA-binding activity from whole-cell extracts (lanes 6 and 7) which migrated faster than p50–p65 (compare lanes 2 and 7) and which was completely supershifted with an anti-p50 antibody (lane 9). Supershifting was blocked in the presence of the specific peptide antigen (lane 8). The Bcl-3-associated DNA-binding activity was not affected by antibodies directed against p52 (lanes 10–13) or p65 (not shown). The IP-shift assay thus allows the detection of distinct NF-κB dimers bound to different members of the IκB family. The same results as those shown above were obtained when NF-κB factors were released from Bcl-3 immunocomplexes by using peptides containing the antibody-binding site instead of detergent (data not shown).

We next examined the cellular distribution of the Bcl-3–p50 complex using nuclear and cytoplasmic extracts prepared from HeLa or Namalwa B cells (Figure 1B, left panel). The Bcl-3–p50 complex could

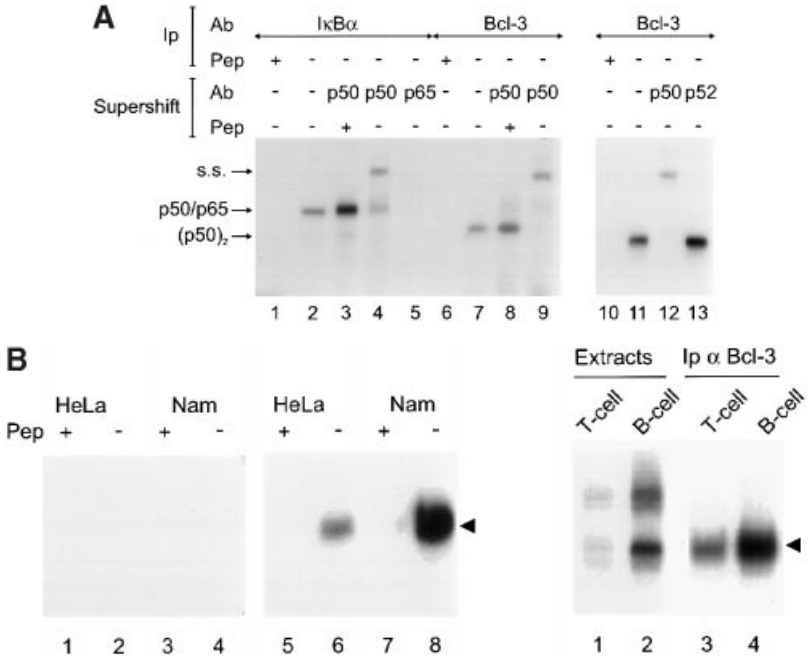


**Fig. 1.** Identification of cellular Bcl-3 complexes. Bcl-3 is associated with p50 but not with p52 in nuclear fractions of cell lines and in primary B and T cells. (**A**) Immunoprecipitation–electrophoretic shift assay (IP-shift assay) to detect NF-κB proteins associated with specific IκB members. Cellular extracts were subjected to immunoprecipitation and analysed for NF-κB/Rel components by detergent elution of the pellets and subsequent EMSA. Immunoprecipitations using HeLa cell extracts were carried out with an anti-IκBα antibody (lanes 1–5) or with an anti-Bcl-3 antibody (lanes 6–13). In lanes 1, 6 and 10, precipitations were performed in the presence of the antibody-specific peptides. Precipitated proteins were detergent-eluted from the pellets and assayed by EMSA with the $H_2K$ NF-κB-binding site as a probe. Antibodies against p50, p65 or p52 were added to the DNA-binding reactions in lanes 3–5, 8, 9, 12 and 13, as indicated. Specificity of the antibody supershifts was monitored by addition of the specific peptides in lanes 3 and 8. Free DNA is not shown. (**B**) Bcl-3–p50 complexes in nuclear extracts of HeLa and Namalwa B cells and in splenic T and B cells. Cytosolic (lanes 1–4) or nuclear extracts (lanes 5–8) of HeLa (lanes 1,2, 5 and 6) or Namalwa cells (lanes 3, 4, 7 and 8) (left panel) or whole-cell extracts of splenic T (lane 3) or B cells (lane 4) (right panel) were subjected to IP-shift assay analysis as described in (A). The peptide specific for the anti-Bcl-3 antibody was added as indicated. In addition, whole-cell extracts of splenic T and B cells (right panel, lanes 1 and 2) were analysed directly by EMSA. The migration of p50 homodimers, liberated from Bcl-3, is indicated by an arrow. Note that under the EMSA conditions used, ternary complexes containing Bcl-3 are not formed.

be immunoprecipitated using nuclear extracts from either cell type (lanes 6 and 8). No DNA-binding activity was recovered from the cytoplasmic extracts (lanes 2 and 4). Interestingly, Namalwa cells contain much higher amounts of Bcl-3–p50 DNA-binding activity than HeLa cells (lanes 5–8), although Namalwa cells do not contain higher amounts of Bcl-3 (data not shown). When comparing primary B and T cells isolated from murine spleens, B cells contain more Bcl-3-associated p50 than T cells (Figure 1B, right panel, lanes 3 and 4).

In contrast to the cytoplasmic IκB molecules, no effect of cellular stimulation has been described for Bcl-3. Therefore, we have analysed whether Bcl-3–p50 complexes are induced by agents that activate NF-κB p50–p65 by using the IP-shift assay. To determine whether Bcl-3–p50 is inducible, the amount of complexes was determined at various time points following TNF-α stimulation (Figure 2A). Bcl-3–p50 complexes were in fact strongly induced by TNF-α in HeLa cells. Compared with unstimulated cells (lane 6), an increase of Bcl-3–p50 was evident after 10 min of TNF-α treatment and peaked after 20 min (lanes 7 and 8). Elevated amounts persisted at least 120 min post-induction (lane 9). The induced activity was specific, as shown by peptide competition for the 20 min time point (lane 5). Thus, the effect of TNF-α on the accumulation of Bcl-3–p50 is a rapid response and follows very similar kinetics when compared with activation of p50–p65 by TNF-α (lanes 1–4). Next, IL-1β, phorbol 12-myristate 13-acetate (PMA) and okadaic acid (OA), all known inducers of p50–p65, were assayed for their effect on the amount of Bcl-3–p50 complexes (Figure 2B). All three agents led to an activation of p50–p65 and to an accumulation of Bcl-3–p50 complexes with the same kinetics. Both species were induced maximally by OA or PMA only after 60 min, whereas IL-1β caused a rapid activation seen at 30 min, which subsequently declined at 60 min (Figure 2B, lanes 7–9).

### *Inducible formation of p50 homodimers requires proteasome activity but is independent of p50–p65 activation*

To rule out that induction of Bcl-3–p50 was an indirect effect due to an exchange of p50 subunits from p50–p65 to Bcl-3, HeLa cells were stably transfected with IκBαΔN or empty vector (Figure 3A). While, as expected, IκBαΔN expression caused a strong reduction of TNF-α-induced p50–p65, accumulation of Bcl-3–p50 complexes was unaffected (compare lanes 2 with 7, and lanes 4 with 9 in upper and lower panels, respectively). Thus, Bcl-3–p50 complexes are not formed from p50–p65 heterodimers, released from IκBα, and hence their induction does not require IκBα degradation. Inhibition of the proteasome by either ALLN or lactacystin blocked formation of Bcl-3–p50 and release of p50–p65 after TNF-α stimulation (Figure 3A and B). Similarly, formation of Bcl-3–p50 complexes induced by OA was inhibited efficiently by

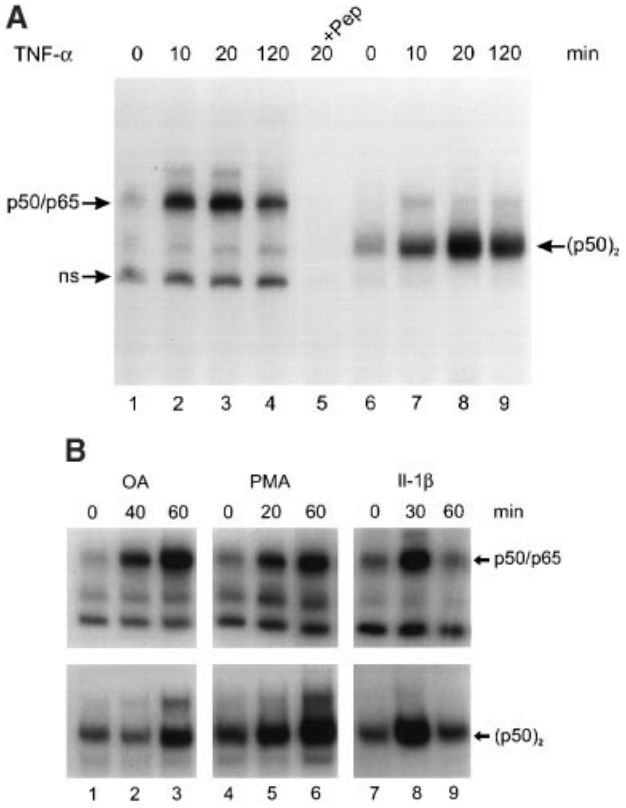


**Fig. 2.** TNF-α, IL-1β, PMA or OA induce release of NF-κB p50–p65 and Bcl-3–p50 complexes with similar kinetics. (**A**) HeLa cells were induced with 20 ng/ml TNF-α for the indicated times. Whole-cell extracts were subjected to EMSA (lanes 1–4) or to IP-shift analysis (lanes 5–9). Immunoprecipitation was performed with (lane 5) or without (lanes 6–9) the specific peptide and eluates were analysed by EMSA using the $H_2K$ site as a probe. Free DNA is not shown. The lower complex is non-specific (ns). (**B**) HeLa cells were treated with 0.5 μM OA, 75 ng/ml PMA or 10 ng/ml IL-1β for the indicated times and extracts were subjected directly to EMSA (top panels) or IP-shift assay (bottom panels). The migration of p50–p65 and p50 homodimers eluted from Bcl-3 is indicated.

ALLN (data not shown). This strongly suggests that the proteosomal degradation machinery acting on p105 is required for the formation of Bcl-3–p50 complexes.

### *p105 is phosphorylated by the IκB kinases IKKα and β in response to TNF-α stimulation*

Due to the kinetics and the proteasome dependency for the inducible formation of Bcl-3–p50 complexes, our data suggest that IκB kinases, which are activated by diverse NF-κB-activating agents (Stancovski and Baltimore, 1997), could directly phosphorylate p105 and thereby induce its ubiquitin-dependent proteolysis. We had shown previously that cellular p105 and IκBα are phosphorylated rapidly with the same kinetics in TNF-α- or hydrogen peroxide-treated cells (Naumann and Scheidereit, 1994). Thus, IκBα wild-type protein, its Ser32,36Ala mutant, p105, p100 and Bcl-3 were expressed in bacteria or with the baculovirus system and compared as IKK substrates in an *in vitro* kinase reaction. Roughly equimolar amounts of the substrates were incubated with anti-HA immunoprecipitates from mock-transfected HeLa cells or HA-IKKα-transfected cells that were either untreated or stimulated for 5 min with TNF-α (Figure 4A). As expected, IκBα was phosphorylated in IKKα-transfected cells and the efficiency was enhanced by TNF-α stimulation (lanes 5 and 6). Phosphorylation depended on serines 32 and 36 since the mutant showed only weak non-specific phosphorylation (lanes 1–3) also observed with wild-type IκBα assayed without transfected IKKα (lane 4). Phosphorylation of p105 by IKKα was as efficient as that of IκBα and could be stimulated equally by TNF-α (compare lanes 5 and 6 with 8 and 9); no phosphorylation was seen without transfected IKKα (lane 7). In contrast to p105, p100 was not an IKK substrate (lanes 10 and 11). This is an interesting observation, since we could not detect any p52 bound to Bcl-3 in TNF-α-induced cells (Figure 1 and data not shown). Recombinant Bcl-3 was strongly phosphorylated by unknown kinases (Figure 4A, lanes 12–14) present even in immune complexes from cells not transfected with IKKα (lane 12). Consistent with this, extensive constitutive phosphorylation of cellular and transfected Bcl-3 has been observed previously (Nolan *et al*., 1993). We could not detect any TNF-α-dependent phosphorylation of Bcl-3 above the level of constitutive phosphorylation (Figure 4B and data not shown). Thus, TNF-α signalling does not appear to act directly on Bcl-3.

To analyse the relative preference of IKKα and IKKβ towards IκBα and p105, a mixture of both substrates was incubated with immune complexes from cells transfected with either kinase (Figure 4B). Whereas both kinases phosphorylated IκBα equally well and showed an equivalent activity increase in response to TNF-α, p105 was a slightly better substrate for IKKα compared with IKKβ (lanes 2–4).

### *Delineation of C-terminal IKK phosphorylation sites in p105*

The IKKα phosphorylation sites on p105 were localized to its C-terminal end by testing various N- and/or C-terminally truncated proteins (Figure 5). Neither p50, corresponding to the N-terminal half of p105 (Figure 5B, lanes 5–6), nor a region from amino acids 202–818 of p105 (lanes 10 and 11) was phosphorylated by IKKα. TNF-α-stimulated and IKKα-dependent phosphorylation was obtained with the complete C-terminal half of p105 (lanes 7–9) and with a construct containing its last 151 amino acids (lanes 12–14).

The phosphorylation sites were narrowed down further with a series of short C-terminal deletions (Figure 5C, see Figure 5A for summary). In brief, all residues of p105 phosphorylated by IKKα are C-terminal to amino acid 850 (ΔC5, lanes 12 and 13). The major sites are located within amino acids 920 and 936 (compare lanes 4 and 5 with lanes 6 and 7). Weak phosphorylation was still observed between amino acids 850 and 891 (lanes 6–13). The major phosphorylation region contains three serines (Ser921, Ser923 and Ser932) and two threonines (Thr927 and Thr931) (Figure 5A). This region is conserved in human, rodent and avian p105, but not in p100, consistent with the observation that p100 is not phosphorylated by IKKα (Figure 4A).

### *IKKα and β stably associate with a C-terminal domain of p105 in intact cells*

To determine if IKKα or IKKβ associate with p105 in intact cells, 293 cells were transfected with FLAG-tagged p105, p50, p105ΔC5 or p105ΔN along with HA-tagged

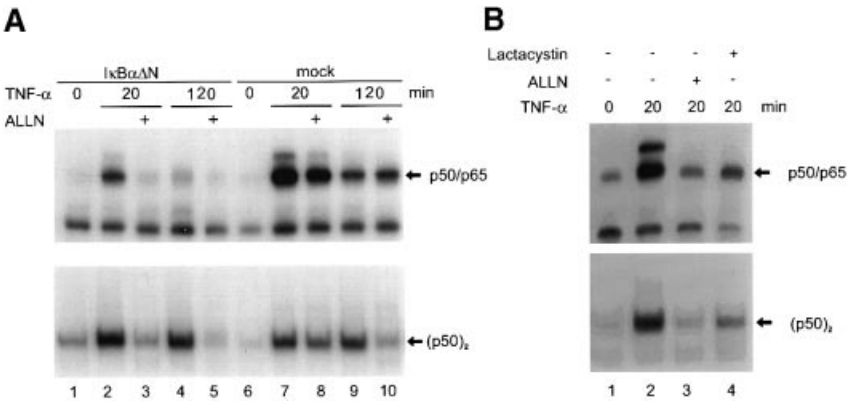


**Fig. 3.** Induction of Bcl-3–p50 complexes is independent of IκBα degradation and p50–p65 release, but is sensitive to proteasome inhibitors. (**A**) HeLa cells, stably transfected with an IκBαΔN expression vector (lanes 1–5) or empty vector (lanes 6–10), were treated with 20 ng/ml TNF-α for various times, with or without pre-incubation with the proteasome inhibitor 50 μg/ml ALLN, as indicated. Extracts were assayed by EMSA (top panel) or IP-shift assay (bottom panel) as described for Figure 2. (**B**) HeLa cells were left untreated (lane 1) or stimulated with 20 ng/ml TNF-α for 20 min (lanes 2–4) without or with pre-incubation with 50 μg/ml ALLN or 50 μM lactacystin, as indicated. EMSA (top) or IP-shift assay (bottom panel) was performed with whole-cell extracts. The slower migrating protein–DNA complex in lanes 7 (A) and 2 (B) represents p65 homodimers (upper panels).

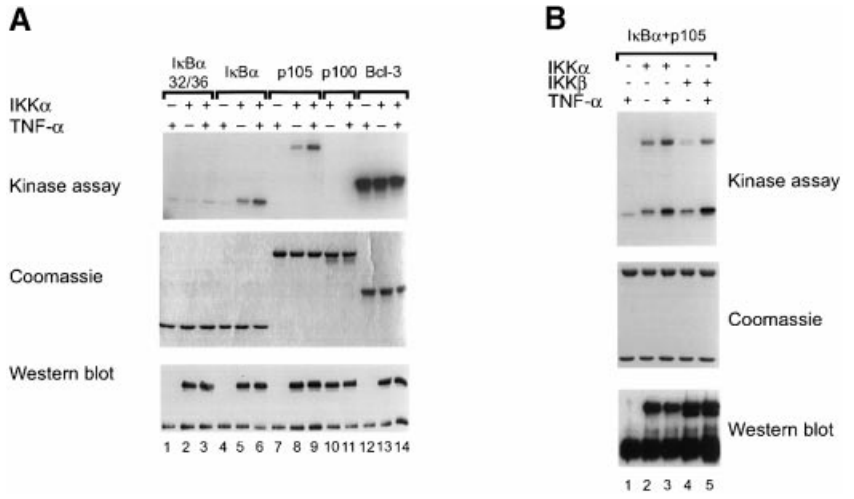


**Fig. 4.** Transfected IKKα and IKKβ phosphorylate IκBα and p105 in response to TNF-α stimulation with similar efficiency. (**A**) Immunocomplex kinase reactions (top panel) were performed with HeLa cells that were mock-transfected, transfected with IKKα and untreated or TNF-α stimulated, as indicated. Phosphorylated proteins were visualized by autoradiography. The roughly equimolar amounts of the recombinant substrate proteins IκBαS32/36A, IκBα, p105, p100 and Bcl-3 used in the assay are shown by Coomassie Blue staining (middle panel). Expression of transfected IKKα was controlled by Western blotting, using the HA tag antibody for IP and the IKKα antibody for immunodetection (bottom panel). (**B**) A mixture of recombinant IκBα and p105 proteins was subjected to immunocomplex kinase reactions with extracts either from mock-transfected, IKKα- or IKKβ-transfected cells that were untreated or stimulated with TNF-α, as indicated. Coomassie Blue staining of the substrate proteins is shown in the middle panel and Western analysis of anti-HA-immunoprecipitated IKKα or IKKβ with anti-HA antibody in the bottom panel. Please note that species-specific immunodetection results in the strong IgG signal in the Western blot (lower band).

IKKα or IKKβ. Expression of these proteins was controlled in Western blots (Figure 6, upper panels). Expression of either p105 or p105ΔC5 resulted in equivalent formation of p50 by processing (upper panels, lanes 1–3, 6 and 7) and this process thus did not require the sequences C-terminal to amino acid 850, containing the IKK substrate sites. Using a FLAG antibody, IKKα and IKKβ were both co-precipitated efficiently with FLAG-p105 (bottom panels, lanes 2 and 3). Subregions of p105 were tested for association with IKKα: FLAG-p105ΔN was as effective as FLAG-p105 in associating with IKKα (compare lanes 2 and 11), whereas FLAG-p50 did not co-immunoprecipitate with IKKα (lanes 8 and 9). FLAG-p105ΔC5 interacted only very weakly with IKKα (lane 7). Thus, strong interaction of IKKα is restricted to p105 derivatives containing the IKK substrate sites.

Similarly to these findings with p105, it has been shown previously that co-expressed IκBα and IKKα associate in 293 cells. In contrast to p105, this interaction required co-transfected p50–p65, presumably to stabilize ectopic IκBα (Regnier *et al.*, 1997). Efficient interaction of IKKα with IκBα or p105 is in accordance with their equivalent phosphorylation (Figure 4A)

### *TNF-α induces p105 degradation; requirement for a destruction box containing the IKK sites*

The finding that TNF-α induces p105 phosphorylation by IKKα (Figures 4 and 5) suggests that this is a rate-limiting step for precursor proteolysis and release of p50 or other associated NF-κB subunits. We have therefore analysed the stability of p105 with or without TNF-α stimulation in a pulse–chase analysis (Figure 7). The half-life of

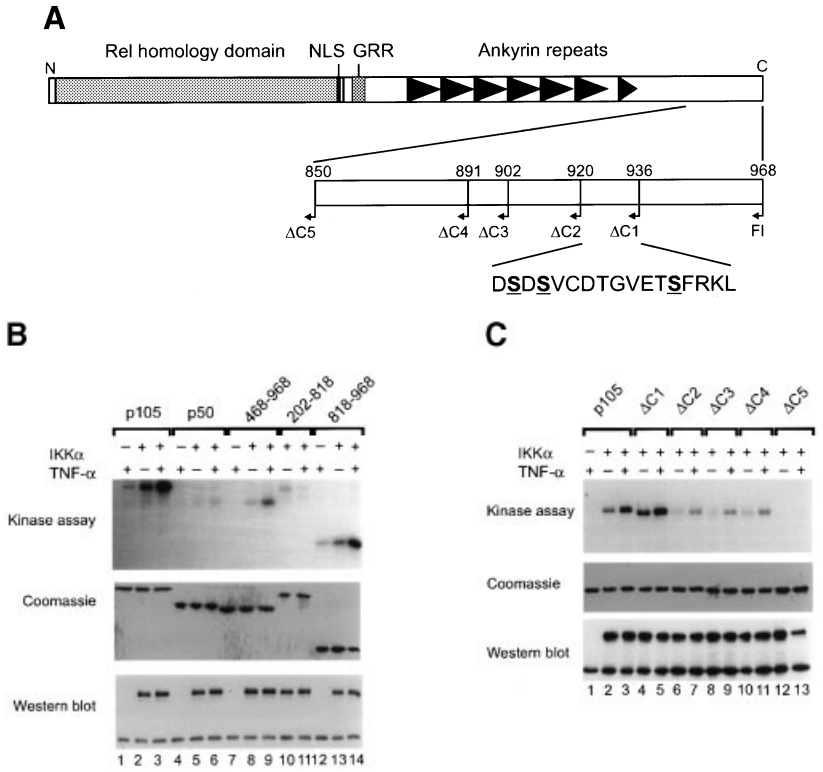


**Fig. 5.** Delineation of the IKKα phosphorylation sites in p105. (**A**) Schematic summary. Location of end-points of deletion mutants and delineated phosphorylation sites in the C-terminal sequence of p105. The Rel homology domain, nuclear localization signal (NLS), glycine-rich region (GRR) and ankyrin repeats (triangles) are indicated. Numbers refer to p105 amino acid positions. Ser923, Thr927 and Thr931 are part of overlapping motifs (DSVCDT and DTGVET) similar to the conserved sequence D(S,T)Gxx(S,T) containing the substrate sites for IKKs in IκBα, β and ε. Bold and underlined residues are IKK phosphorylation sites mapped by point mutagenesis. The sequence between ΔC1 and ΔC2 is highly conserved between human, mouse, rat and chicken p105. Amino acid conservation outside this region is considerably less (not shown). (**B**) Full-length p105 and p105 deletion mutants (p50, amino acids 1–368, the C-terminal half of p105, an N- and C-terminally truncated p105 and the last 150 amino acids of p105, encoded amino acids are indicated) were produced in bacteria and tested in immunocomplex kinase reactions (top panel) with extracts from IKKα- or mock-transfected cells that had been treated with TNF-α or left untreated, as indicated. Substrate proteins are shown in the middle panel (Coomassie Blue staining) and IKKα expression is shown in the bottom panel (Western blot of immunoprecipitated IKKα). (**C**) Full-length p105 and C-terminal deletion mutants (see A for summary) were assayed for IKKα phosphorylation as described in (B). Please note that the size differences between the p105 mutants are not resolved in this short run of the SDS–gel.

endogenous p105 was significantly reduced in TNF-α-stimulated cells (Figure 7A), as shown earlier using a different experimental setting (Naumann and Scheidereit, 1994). p50 accumulated slightly during the chase period in unstimulated cells, presumably due to post-translational processing. Likewise, the turnover of transfected p105 with a half-life of ~90 min in untreated cells was augmented to <20 min in the presence of TNF-α (Figure 7B). In contrast, p105ΔC3 and p105ΔC5, lacking the major IKKα phosphorylation sites, were strongly stabilized and the turnover of the mutants was not increased by TNF-α stimulation. Furthermore, no consistent strong formation of p50 was observed (not shown), as would be expected for induced p105 processing. This suggests that complete degradation of p105, rather than processing, results in release of associated Rel factors.

### *A p105 mutant lacking the destruction box is a super-repressor of IKK-induced NF-κB activity*

If p105 proteolysis by site-specific, signal-induced phosphorylation controls NF-κB signalling, p105 should act as a dominant-negative regulator if devoid of its IKK phosphorylation sites, as has been shown for IκBα (Brown *et al*., 1995; Traenckner *et al*., 1995; Whiteside *et al*., 1995). To address this point, we first compared IKK-induced phosphorylation of p105 and IκBα in intact cells. Expression constructs encoding p105ΔN or p105ΔNΔC5, IκBα or IκBαΔN were transfected into 293 cells either alone or together with IKKα or IKKβ (Figure 8A). As expected, a phosphorylated, slower migrating band of IκBα was observed in the presence of either IKK (lanes 7–9). The C-terminal half of p105 (p105ΔN) was phosphorylated with an efficiency comparable with IκBα, also resulting in a retarded band (lanes 1–3). In contrast, neither IκBαΔN nor the p105ΔNΔC5 mutant gave rise to phosphoforms (lanes 4–6 and 10–12). These results confirmed the IKK phosphorylation data obtained *in vitro* (Figures 4 and 5).

When the cells were co-transfected with an NF-κB reporter plasmid, expression of IKKα or IKKβ led to a 10-fold induction of reporter activity (Figure 8B). Expression of p105ΔN or IκBα resulted in slightly reduced IKKα- or β-stimulated reporter activity, whereas p105ΔNΔC5 or IκBαΔN significantly repressed induced NF-κB activity. The difference between the inhibitors and their dominant-negative mutants was more pronounced in IKKβ-transfected cells. Whereas in the presence of p105ΔN or IκBα a 6-fold activation was still seen, expression of either p105ΔNΔC5 or IκBαΔN nearly abolished the stimulating effect of IKKβ (Figure 8B).

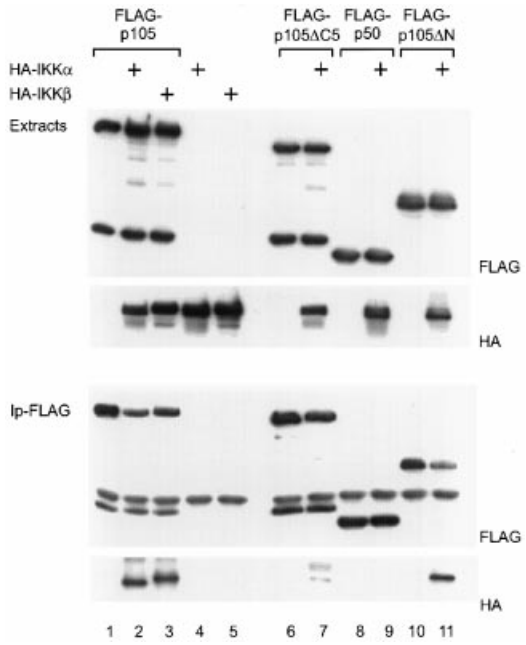


**Fig. 6.** p105 physically associates with IKKα and IKKβ in intact cells. 293 cells were transfected with FLAG-tagged versions of p105, p105ΔC5, p50 or the C-terminal half of p105 (p105ΔN), respectively, and with HA-tagged IKKα or IKKβ, as indicated. Whole-cell extracts were tested for expression of transfected proteins in a Western blot using the FLAG and HA antibodies, respectively (upper two panels). Extracts were subjected to immunoprecipitation using the FLAG antibody. Precipitated p105 and p105 mutants were revealed by Western blotting with the FLAG antibody and co-precipitated IKKα and IKKβ were detected with the HA antibody (lower two panels). Please note that the slower migrating signal in lane 7 is a cross-reacting product.

This experiment demonstrates the functional equivalence between p105 and IκBα. Both molecules can only marginally repress NF-κB activity when overexpressed, since both are degraded following stimulation. In each case, the removal of a degradation box, containing IKK phosphorylation sites, results in stabilized super-repressor molecules which sequester NF-κB. The data indicate that NF-κB–Rel complexes of p105 or IκBα are equivalent downstream targets of the IKK complex.

### *Three serine residues in the C-terminal destruction box of p105 are IKK complex phosphoacceptor sites and are required for TNF-α-induced degradation*

To determine IKK phosphoacceptor residues in p105, serines and threonines in the C-terminus were substituted by alanine. Surprisingly, combined mutation of Ser923, Thr927 and Thr931, which are part of two overlapping motifs similar to the DSGψXS phosphorylation site in IκBα (Figure 5A), did not result in diminished phosphorylation of p105 by IKKα (data not shown). However, mutation of serines 921, 923 and 932 to alanines strongly reduced both basal and TNF-α-induced phosphorylation of p105ΔN by IKKα (Figure 9A, compare lanes 2 and 3 with lanes 5 and 6). Some weak phosphorylation still observed with the alanine mutant is likely to occur in between amino acids 850 and 891, consistent with the data shown in Figure 5C. The identified IKK phosphorylation sites are within the boundaries of the deletion mutants ΔC1 and ΔC2 (Figure 5A). Phosphorylation at these sites is thus in agreement with the requirement of this region for IKK-mediated p105 proteolysis. However, it cannot be ruled out that IKK binding *per se* to p105 is required for signal-induced degradation and that p105 phosphorylation is a separate event. To address this point, p105ΔN and p105ΔNS921A/S923A/S932A were stably transfected into HeLa cells and tested for stability after TNF-α treatment (Figure 9B). Endogenous IκBα was analysed as a control in all clones. It was degraded after TNF-α treatment and stabilized by ALLN as a slower migrating phosphorylated isoform (lanes 1–12). Likewise, p105ΔN was degraded efficiently after TNF-α stimulation, while inhibition of the proteasome by ALLN blocked degradation, resulting in a stabilized hyperphosphorylated protein with retarded migration (lanes 4–6). In contrast, mutation of serines 921, 923 and 932 completely stabilized p105ΔN in the presence of TNF-α in two independent cell clones (lanes 7–9 and 10–12) and ALLN had no discernible effect. Thus, TNF-α-mediated p105 degradation requires the identified IKK phosphorylation sites.

## Discussion

The precursor molecules p105 and p100 retain NF-κB subunits in the cytosol (Rice *et al*., 1992; Mercurio *et al*., 1993; Naumann *et al*., 1993a,b) and are critical for the generation of p50 and p52 homodimers, which are bound specifically by the predominantly nuclear IκB homologue Bcl-3. It was unclear how, and under which conditions, precursors respond to signalling pathways that activate NF-κB and whether Bcl-3-containing complexes are subject to regulation by such pathways. Specifically, it was ambiguous as to whether precursor complexes can be activated directly by proteolysis following cellular stimulation. In this work, we have investigated the effect of known NF-κB-inducing agents on the production of Bcl-3–Rel complexes, on precursor phosphorylation by IκB kinases and on precursor stability.

The analysis of p105- or p100-derived NF-κB/Rel factors by signal-induced processes is complicated by the fact that both precursors bind to factors, such as p65 or c-Rel, which also associate with IκBα, β or ε (Verma *et al*., 1995; May and Ghosh, 1997). Bcl-3-bound p50 dimers are in fact a suitable readout system for p105-controlled Rel activity: in contrast to Bcl-3, neither IκBα, β nor ε binds to p50 efficiently and p50 is produced from processed p105 and sequestered by p105. To detect Bcl-3-associated p50, we developed an IP-shift assay which involves precipitation of complexes with an anti-Bcl-3 antibody and subsequent analysis of NF-κB/Rel activities, released from Bcl-3, by EMSA. Apart from its specificity, this procedure has the advantage that protein complexes which are of low abundance, and would be overshadowed by more abundant complexes in EMSA analysis using crude cellular extracts, can be analysed specifically.

By using this IP-shift analysis with antibodies against Bcl-3, we could demonstrate that cellular Bcl-3 is associated with p50 but not with p52 homodimers. This selectivity may be explained by the lower expression of p52/p100 compared with p50/p105 and by less efficient processing of p100 to p52 (Betts and Nabel, 1996) in the cell types analysed. In accordance with earlier reports, we found that

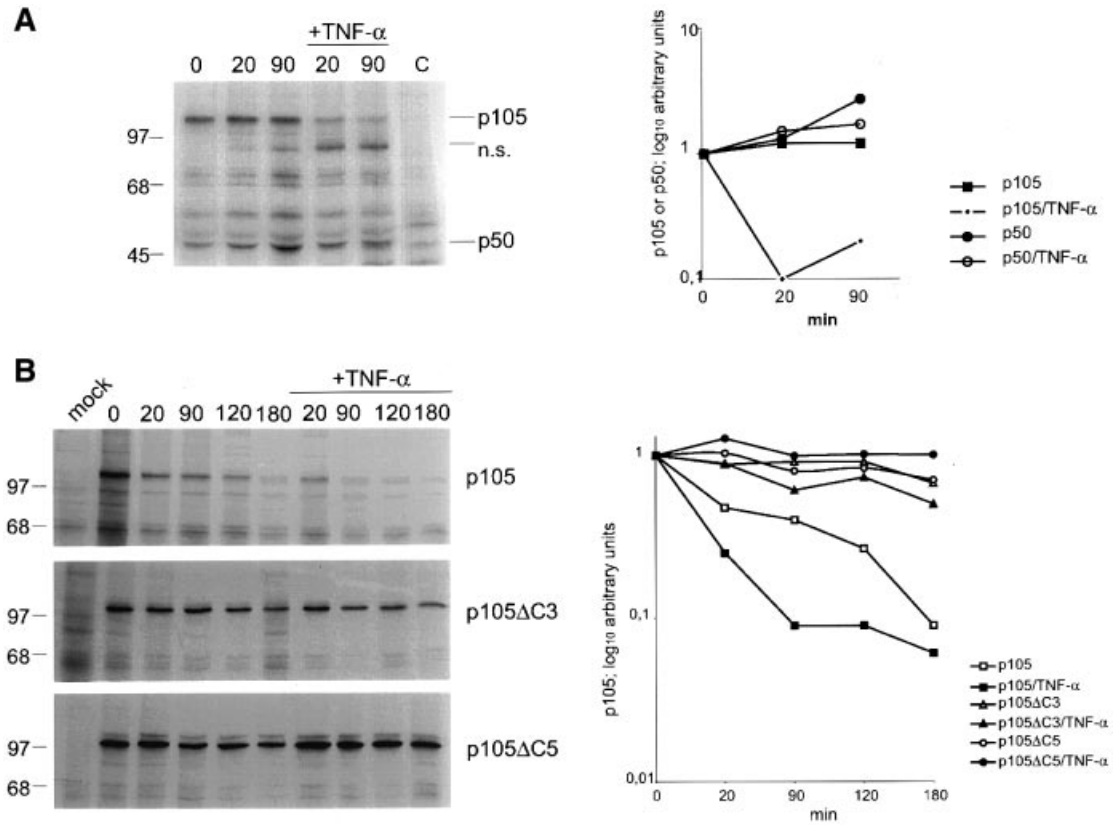


**Fig. 7.** TNF-α-induced p105 degradation; requirement for a C-terminal destruction box. (**A**) HeLa cells were labelled with [$^{35}$S]methionine/[$^{35}$S]cysteine for 4 h and chased with cold medium without or with TNF-α stimulation for the times indicated (min). Endogenous p105/p50 was immunoprecipitated with an antibody directed against an N-terminal epitope, resolved by SDS–PAGE and visualized by autoradiography. The migration of p50 and p105 is indicated. (**B**) HeLa cells were stably transfected with wild-type p105, p105ΔC3 or p105ΔC5 and metabolically labelled with [$^{35}$S]methionine for 1 h. Cells were left untreated or chased with cold medium with or without TNF-α for the indicated times. Transfected proteins were precipitated with the anti-FLAG epitope antibody and visualized after SDS–PAGE by autoradiography. Quantitations after densitometric scanning of the signals are shown on the right of (A) and (B), respectively.

Bcl-3–p50 complexes are more abundant in transformed or primary B cells than in non-B cells (Caamano *et al*., 1996; Watanabe *et al*., 1997).

The amount of Bcl-3–p50 complexes was increased rapidly following stimulation with TNF-α, IL-1β, PMA or OA, in each case following the same inducer-specific kinetics as activation of NF-κB p50–p65. The strikingly similar kinetics suggest that Bcl-3–p50 activity is controlled by the IκB kinase complex. Since activation of Bcl-3–p50 was blocked by proteasome inhibitors, but not by expression of IκBαΔN, which blocks release of p50–p65 following IκBα degradation, it likely requires precursor proteolysis. Using *in vitro* kinase assays as well as analysis of transfected molecules, we found that both p105 and IκBα are phosphorylated inducibly by IKKα or IKKβ with similar efficiencies. In contrast, p100 was not phosphorylated by either kinase. This is surprising, since endogenous p100 and p105 were both phosphorylated in TNF-α-stimulated cells with the same kinetics as endogenous IκBα (Naumann and Scheidereit, 1994). The TNF-α-stimulated kinase(s) acting on p100 thus remains to be identified.

For Bcl-3, we detected constitutive phosphorylation, independent of IKKs. We were unable to detect phosphorylation of Bcl-3 following TNF-α stimulation. We did not observe increased phosphorylation of Bcl-3 in HeLa cells after stimulation with TNF-α or upon overexpression of IKKs (data not shown). Thus, Bcl-3 does not appear to be a target for TNF-α-induced protein kinases.

IKKα and β phosphorylation sites were delineated in the C-terminus of p105 by deletion analysis to a short stretch of 17 amino acids containing three serine and two threonine residues. A region encompassing the phosphorylation sites was necessary to confer stable association of p105 with IKKα or IKKβ in 293 cells. Under similar conditions, association has been shown between IKKα and IκBα (Regnier *et al*., 1997), underscoring equivalent substrate utilization of p105 and IκBα by both IKKs. Point mutagenesis revealed that serines 921, 923 and 931 are the substrate sites of the IKK complex. Ser923, Thr927 and Thr931 are part of two phylogenetically conserved overlapping motifs related to the DSGψXS motif embedding IKK substrate sites in IκBα, β or ε (Figure 5A). However, combined mutation of these residues had no effect on p105 phosphorylation. This is in accordance with earlier reports that IKKα and β show a strong preference to phosphorylate serine over threonine residues (DiDonato *et al*., 1997; Mercurio *et al*., 1997; Li *et al*., 1998). In IκBα, the DSGψXS motif, when phosphorylated by IKKs, serves as a recognition site for β-TrCP, a component of the ubiquitin ligase (Yaron *et al*., 1998; Spencer *et al*., 1999). A similar motif in β-catenin, phosphorylated by glycogen synthase kinase 3β (GSK3β), is also bound by β-TrCP (Winston *et al*., 1999), but IKKs

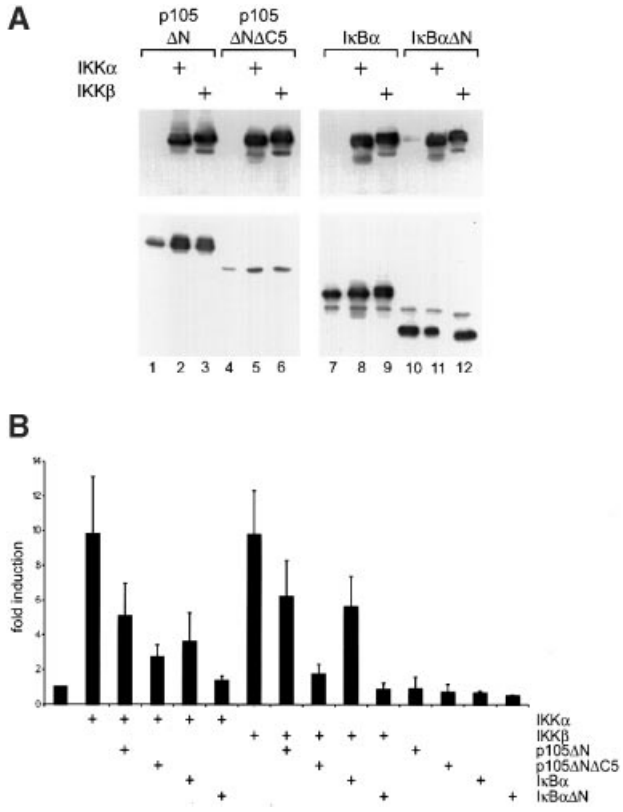


**Fig. 8.** A p105 mutant lacking the C-terminal destruction box acts as a dominant-negative inhibitor of IKKα- or β-induced NF-κB reporter gene activity in a similar way to N-terminally truncated IκBα. (**A**) 293 cells were transfected with the C-terminal half of p105 either containing (p105ΔN) or not containing (p105ΔNΔC5) the IKK phosphorylation sites, or with full-length IκBα or IκBΔN, lacking the N-terminal destruction box. IKKα and IKKβ were co-transfected or not, as indicated. Whole-cell extracts, prepared in the presence of phosphatase inhibitors, were analysed in Western blots for expression and phosphorylation of transfected proteins. IKKs (top panels) were detected with an HA antibody, p105ΔN, IκBα and deletion mutants were detected with a FLAG antibody. (**B**) 293 cells were transfected with IκB molecules and IKKs, as indicated, and additionally were co-transfected with a 2 × NF-κBluc reporter plasmid (Hirano *et al.*, 1998). Luciferase activity was measured and standardized by co-transfection of pRLTKluc expression vector. Results represent the mean values of at least three independent experiments and standard deviations are indicated.

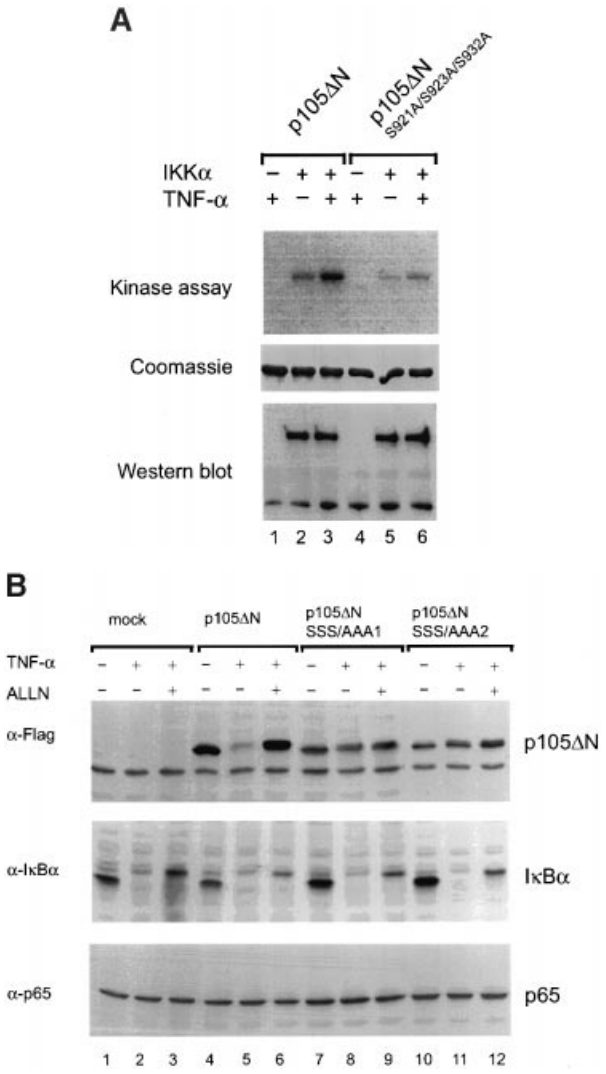


**Fig. 9.** Identification of IKK phosphoacceptor sites in p105. (**A**) p105ΔN and p105ΔNS921A/S923A/S932A were expressed in *Escherichia coli* and assayed in immunocomplex kinase reactions (top panel) as described in Figure 5B. Middle and bottom panels: substrate proteins and IKKα expression, respectively. (**B**) Phosphorylation at serines 921, 923 and 932 is required for TNF-α-induced degradation of p105ΔN. HeLa cells were stably transfected with an empty expression vector (mock), or with FLAG-p105ΔN or FLAG-p105ΔNS921A/S923A/S932A (p105ΔNSSS/AAA) expression constructs. Cells were treated with 20 ng/ml TNF-α for 15 min with or without 50 μg/ml ALLN pre-incubation or were left untreated, as indicated. For the triple serine mutant, two different stable clones were analysed (SSS/AAA1 and SSS/AAA2). Transfected p105 was visualized by anti-FLAG antibody and endogenous IκBα and p65, the latter as equal loading control, by the respective antibodies in a Western blot.

do not inducibly phosphorylate β-catenin (data not shown). Thus, the DSGψXS motif does not appear to be sufficient for specific substrate recognition of IKKs. It remains to be determined which conserved substrate sequences determine IKK specificity. A further question to be resolved is whether β-TrCP or a different F-box protein binds to p105 after its phosphorylation by the IKK complex. An interaction of p105 with a ubiquitin ligase is a likely possibility since the mapped phosphorylation sites in the C-terminus conferred induced degradation.

We found that deletion of the IKK sites did not interfere with p105 processing (Figure 6). In earlier studies, we and others proposed that p105 processing is inducible (Mellits *et al*., 1993; Mercurio *et al*., 1993; Naumann and Scheidereit, 1994; Donald *et al*., 1995; MacKichan *et al*., 1996). However, the increase of p50 detected after stimulation with agents including TNF-α and PMA was variable, whereas a pronounced decline of p105 was observed consistently. Thus, it appears that complete degradation rather than processing may be a prevailing mechanism induced by signalling and that processing is a constitutive process. In line with this, Belich *et al*. (1999) observed strong and complete p105 degradation and not increased processing in cells stimulated with TNF-α or overexpressing Tpl-2 (see below). Likewise, p105 undergoes degradation in LPS stimulated monocytes without enhanced generation of p50 by processing (Harhaj *et al*., 1996).

The stabilization of p105 in TNF-α-stimulated cells upon removal of the C-terminal sequence is similar to

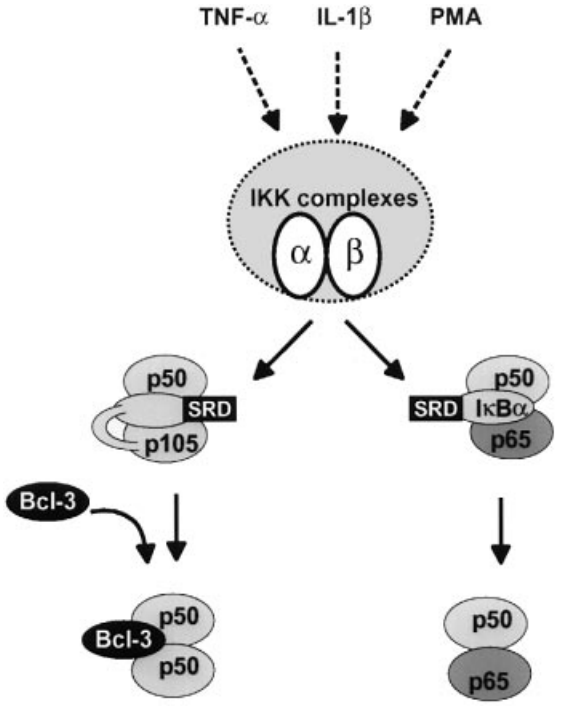


**Fig. 10.** The data presented are summarized schematically. Depending on relative expression levels, p105 is associated predominantly with p50, generated by constitutive processing, or with other NF-κB subunits. Following activation by diverse agents, the IKK complex is activated and phosphorylates p105 and IκBα at their C- and N-terminal destruction boxes, respectively, resulting in complete degradation of both inhibitors. Released p50 is sequestered by Bcl-3. The relative amounts of activators set free (e.g. Bcl-3–p50 versus p50–p65) by signalling through the IKK complex is determined by composition and abundance of the different IκB complexes.

the stabilization of IκBα by removal of its N-terminal sequences containing IKK phosphorylation sites at serines 32 and 36, as well as lysines 21 and 22, the major ubiquitin conjugation sites (Brockman *et al*., 1995; Brown *et al*., 1995; Scherer *et al*., 1995; Traenckner *et al*., 1995; Whiteside *et al*., 1995; Baldi *et al*., 1996). The functional similarity of the signal response domains of p105 and IκBα and the equivalence of both molecules in IKK-induced NF-κB activity was demonstrated in a reporter assay. Only upon deletion of their respective N- or C-terminal destruction boxes were IκBα or p105 able to repress IKK-induced NF-κB activity fully.

Our data thus suggest that NF-κB-activating pathways bifurcate downstream of the IKK complex to IκBα and p105 complexes (Figure 10). Following stimulation by diverse signal pathways, the IKK complex is activated and phosphorylates p105 and IκBα at their C- and N-terminal destruction boxes, respectively. Phosphorylation by the IKK complex results in complete degradation of both inhibitors. p105 degradation causes the release of sequestered NF-κB/Rel factors. Depending on the composition and abundance of the various NF-κB and IκB components, different activators are released from p105. For example, processing of p105 results in formation of p105–p50 complexes, giving rise to p50 after degradation of p105. p50 dimers subsequently will be bound to Bcl-3. p105–p65 or p105–c-Rel complexes give rise to dimers containing p65 or c-Rel following loss of p105. It is possible that the responsiveness to IKK-triggered degradation of specific complexes is regulated further by phosphorylation of the Rel subunits either by IKKs or other kinases. In this respect, it has been reported that p65 is phosphorylated efficiently *in vitro* by recombinant IKKs (Mercurio *et al*., 1999) and endogenous p65, following TNF-α stimulation, by unknown kinases (Naumann and Scheidereit, 1994). Furthermore, in *Drosophila*, phosphorylation of both Dorsal and Cactus is required for Dorsal activation (Drier *et al*., 1999).

Since a significant proportion of total p50 but less p65 is associated with p105, p105 plays a unique role in controlling p50 dimer activity (Ishikawa *et al*., 1996). Bcl-3 binds to p50 homodimers released from cytoplasmic pools of p105–p50 heterodimers and migrates with these homodimers to the nucleus (Watanabe *et al*., 1997). IKK-triggered proteolysis of p105 thus regulates the amount of Bcl-3–p50.

The demonstration in this work that Bcl-3–p50 complexes are activated by NF-κB signalling pathways might account for some of the defects observed in mutant mice. In fact, mice deficient in p105/p50 (Sha *et al*., 1995) or Bcl-3 (Franzoso *et al*., 1997; Schwarz *et al*., 1997) display partially overlapping phenotypes, as sharply reduced generation of antigen-specific antibodies and reduced clearance to *Toxoplasma gondii*, *Leishmania monocytogenes* or *Streptococcus pneumoniae* infection.

It was shown recently that rat Tpl-2, a member of the MAP3K kinase family, physically associates with p105 (Belich *et al*., 1999). Tpl-2 overexpression caused increased phosphorylation and degradation of p105. However, no direct phosphorylation of p105 by Tpl-2 could be demonstrated. Curiously, expression of a kinase-inactive Tpl-2 mutant blocked TNF-α-induced degradation of p105 but it did not affect IκBα phosphorylation and degradation. However, in a contrasting report, Cot, the human Tpl-2 homologue, was shown not to be involved in TNF-α induction of NF-κB/Rel activity (Lin *et al*., 1999). Transfected kinase-inactive Cot/Tpl-2 blocks CD3/CD28-stimulated, but not TNF-α-induced NF-κB activity. Lin *et al*. (1999) demonstrated that Cot/Tpl-2 interacts with NIK and IKKα and that it phosphorylates NIK. This places Cot/Tpl-2, like the other MAP3Ks involved in NF-κB activation, NIK and MEKK1 (Malinin *et al*., 1997; Lee *et al*., 1998), upstream of IκB kinases. Our data are in agreement with the model proposed by Lin and colleagues. However, it cannot be excluded that degradation of p105 could be enhanced by Cot/Tpl-2 acting in parallel to the IKKs, affecting still other kinases. Cot/Tpl-2 in fact activates several signalling pathways, including ERK, JNK and NF-AT (Patriotis *et al*., 1994; Salmeron *et al*., 1996; Tsatsanis *et al*., 1998).

The data reported here provide evidence that the p105 precursor and small IκBs, such as IκBα, are equivalent targets of the IKK complex. This implies that various agents and pathways known to activate IκB kinases result in release of NF-κB/Rel not only from small IκBs, but also from p105. Thus, depending on the cell type-specific abundance of the different inhibitor complexes, transcriptional activators other than classical p50–p65, such as Bcl-3–p50, will be activated rapidly by the same signalling pathways. This strongly suggests the involvement of inducible Bcl-3–p50 complexes, and not only p50–p65,

in diverse NF-κB signalling-controlled processes such as apoptosis, cellular proliferation and the immune response. Specifically, it is tempting to speculate that cytokine-induced Bcl-3–p50, like p50–p65, is involved in regulating genes that protect cells from apoptosis.

## Materials and methods

### *Cell culture*

Adherent HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate and 100 U/ml penicillin/streptomycin. Stable cell lines were established after transfection using pcDNA3 expression vector. Cells were selected and clonal cell lines were grown using 600 μg/ml G418. Suspension HeLa (sHeLa) cells were grown in SMEM, 1% non-essential amino acids, 100 μg/ml penicillin/streptomycin and 10% FCS. Namalwa cells were grown in RPMI 1640, 100 μg/ml penicillin/streptomycin, containing 4 mM L-glutamine and 10% FCS. Primary mouse splenocytes were cultured for 16 h in RPMI, 10% FCS, 1% non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B.

### *Purification of splenic B and T cells*

Spleens from nine C57B6 mice were homogenized and lymphocytes isolated by sedimentation in ficoll paque (Pharmacia). MACS was performed according to the manufacturer's protocol, using B220 and Thy1 microbeads and VS+ columns (Miltenyi Biotec). Cell sorting was confirmed by FACS. B cells were >96% and T cells >78% pure. A total of $5 \times 10^6$ cells were used for immunoprecipitation.

### *Transfection and luciferase assay*

Transfections of adherent HeLa cells were carried out with lipofectamine (Gibco-BRL) according to the manufacturer's protocol. 293 cells were transfected by the calcium phosphate precipitation method. For reporter assays, 293 cells were seeded on 60 mm plates and transfected using 5 μg of total DNA. The following DNA amounts were transfected: 200 ng of 2 × NF-κBluc (Hirano *et al*., 1998) as a reporter, 100 ng of pRL-TKluc as an internal control, 800 ng of either IKKα or IKKβ and 200 ng of p105ΔN/p105ΔNΔC5 or 20 ng of IκBα/IκBαΔN, respectively. At 24 h after transfection, cells were lysed and assayed with the dual luciferase kit (Promega) according to the manufacturer's protocol.

### *Antibodies*

Polyclonal antibodies against Bcl-3 (C-14), IκBα (C-21), p65 (A) and HA (Y-11) were obtained from Santa Cruz, p50 and p52 antibodies were from Rockland and Upstate Biotechnology, respectively. Monoclonal FLAG antibody (M2) was purchased from Kodak IBI and monoclonal IKKα antibody B78-1 was purchased from Pharmingen.

### *Plasmids*

p105 constructs were generated by PCR from p105 in pBluescript (Meyer *et al*., 1991). p105 deletion mutants ΔC1(1–936), ΔC2(1–920), ΔC3(1–902), ΔC4(1–891) or ΔC5(1–850) were cloned via *Bgl*II–*Xho*I sites into pcDNA3/FLAG and for bacterial expression via *Bam*HI–*Xho*I sites in pRSETB. p105(202–818) and p105(818–968) were obtained by cloning the respective *Pst*I fragments of p105 into pRSETC. p105ΔN(436–968) and p105ΔNΔC5(436–850) were generated by PCR and cloned into *Bam*HI–*Xho*I sites of pcDNA3/FLAG. p105ΔN(436–968)S921A/S923A/S932A and p105(20–968)S923A/T927A/T931A were generated from the parental pcDNA3/FLAG constructs using the specific primers. All PCR constructs were verified by sequencing. Eukaryotic expression constructs for IκBα and IκBαΔN have been described in Krappmann *et al*. (1996). Bacterial expression constructs for p105(468–968), p50(1–368) and p100 have been described previously (Wulczyn *et al*., 1992; Hatada *et al*., 1993; Naumann *et al*., 1993a). Bcl-3 cDNA was ligated into the *Bam*HI–*Bgl*II sites of pacGHLT-C, that had been modified to encode a precision protease cleavage site. IκBα and IκBαS32/36A inserts were cloned into pGEX-6P1.

### *Protein expression and purification*

IκBα and IκBαS32/36A were expressed from pGEX-6P1 as GST fusion proteins. GST–Bcl-3 was expressed with the baculovirus system. Cleavage by the precision protease (Pharmacia) removed the GST fusion part of all three proteins which were purified further by gel filtration and ion exchange chromatography. All other proteins were purified from preparative SDS–PAGE, eluted, precipitated as described (Hager and Burgess, 1980) and denatured in 8 M urea followed by a stepwise dialysis to renature the proteins.

### *In vitro kinase assay*

Cells were transfected with HA-tagged IKKα or β and stimulated 36 h later with 20 ng/ml TNF-α for 5 min. Cell lysis and immunoprecipitation was done for 1 h in 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100 and 10% glycerol including 1 mM dithiothreitol (DTT), 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate, 0.4 mM pefablock, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. The precipitates were washed twice in cell lysis buffer and once in the kinase reaction buffer containing 20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 20 mM β-glycerolphosphate, 10 mM *p*-nitrophenylphosphate, 50 μM orthovanadate, 1 mM DTT and 20 μM ATP. Kinase reactions were performed with 1 μg of recombinant substrate protein and 5 μCi of [γ-$^{32}$P]ATP in a 15 μl reaction volume. Gels were dried and exposed for 10 min to Kodak X-Omat films. In parallel, the same immunoprecipitations were analysed for precipitated IKK proteins by Western blotting. The amounts of recombinant substrate proteins were visualized by SDS–PAGE and Coomassie Blue staining.

### *IP-shift analysis and EMSA*

Either 800 μg of nuclear and cytoplasmic extracts (Dignam *et al*., 1983) or whole-cell extracts of $5 \times 10^6$ HeLa cells, prepared in lysis buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA or 100 μM KCl for Dignam extracts, 0.1 mM EGTA and 1% NP-40), were adjusted to 100 mM NaCl and 1% NP-40 and were pre-cleared with protein A–Sepharose for 1 h. The supernatant was incubated with the appropriate antibody overnight and immunoprecipitates were collected, washed five times in Dignam buffer D, 1% NP-40, and once in buffer D. Detergent elution was done for 15 min on ice in buffer D, 0.8 % DOC, and the resulting supernatant adjusted to 1.2% NP-40. A 10 μl aliquot of the eluate was used in a standard electrophoretic mobility shift assay using 10 000 c.p.m. of $^{32}$P-labelled $H_2K$ probe.

### *Co-immunoprecipitations*

293 cells grown on 9 cm plates were transfected with 6 μg of HA-tagged IKK construct and 6 μg of FLAG-tagged p105 constructs in a total of 12 μg of DNA. Cells were lysed in a buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 0.4 mM pefablock, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. A quarter of the extracts was used to immunoprecipitate the FLAG-tagged proteins. After incubation for 3 h with FLAG antibody in lysis buffer, the beads were washed four times with lysis buffer, boiled in SDS loading buffer, separated by 9% SDS–PAGE and analysed by Western blotting.

### *Metabolic labelling of cells and immunoprecipitation*

HeLa cells, untransfected or stably expressing p105, p105ΔC3 or p105ΔC5, were pulse-labelled with 170 μCi/ml [$^{35}$S]methionine/[$^{35}$S]cysteine (promix, Amersham Life Science) or 100 μCi/ml [$^{35}$S]methionine, respectively, essentially as described (Krappmann *et al*., 1996). After labelling for the times indicated, the cells were washed once and chased for 0, 20, 90, 120 or 180 min with DMEM (10% FCS) with or without 20 ng/ml TNF-α. Cell extracts were prepared in RIPA buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM DTT, 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate, 0.4 mM pefablock, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A). After 1 h pre-clearing with protein A–Sepharose (Pharmacia), immunoprecipitations were done with anti-FLAG or anti-p50 antibody for 3 h and subsequent collection of immunoprecipitates with protein A–Sepharose for 1 h. The beads were washed five times in RIPA buffer, boiled in sample buffer and eluates were separated by 9% SDS–PAGE. The gels were fixed and incubated in 'amplify' solution (Amersham Life Science), dried and subjected to autoradiography for 48 h at –70°C.

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